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The Polysaccharide Portion Plays an Indispensable Role in *Salmonella* Lipopolysaccharide-Induced Activation of NF-κB through Human Toll-Like Receptor 4

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The lipid A portion has been identified as the active center responsible for lipopolysaccharide (LPS)-induced macrophage activation. However, we found that *Salmonella* (*Salmonella enterica* serovars Abortusequi, Minnesota, and Typhimurium) lipid A is inactive in human macrophages, despite its LPS being highly active. Thus we investigated the critical role of polysaccharide in *Salmonella* LPS-induced activation of NF-κB. In human monocytic cell line THP-1, *Salmonella* lipid A and synthetic *Salmonella*-type lipid A (516) did not induce NF-κB-dependent reporter activity up to 1 μg/ml, whereas strong activation was observed in response to *Salmonella* LPS. The difference in activity between this lipid A and LPS was further examined by using 293 cells expressing human CD14/Toll-like receptor 4 (TLR4)/MD-2, and similar results were obtained in these cells as well. A polysaccharide preparation obtained from *Salmonella* LPS was inactive in 293 cells expressing human CD14/TLR4/MD-2 even in combination with 516. *Salmonella enterica* serovar Minnesota Re LPS, whose structure consists of lipid A and two molecules of 2-keto-3-deoxyoctonic acid, but not its lipid A exhibited strong activity in THP-1 cells and 293 cells expressing human CD14/TLR4/MD-2. These results indicate that the polysaccharide portion covalently bound to lipid A plays the principal role in *Salmonella* LPS-induced activation of NF-κB through human CD14/TLR4/MD-2.

Bacterial lipopolysaccharide (LPS) is a constituent of the outer membrane of the cell wall of gram-negative bacteria and plays a major role in septic shock in humans (25, 30). LPS is composed of a hydrophilic polysaccharide portion and a hydrophobic domain known as lipid A (25). Lipid A species from *Escherichia coli* and *Salmonella* species have been well characterized by both their structure and biological activity (2, 3, 7, 12–14), and both (compounds 506 and 516, respectively) have been synthesized chemically (10, 11, 15). Study of the structure-activity relationship of chemically synthesized lipid A analogues revealed high activity of both the *E. coli*- and *Salmonella*-type lipid A molecules in all test systems examined at the time, although the activity of *Salmonella*-type lipid A was slightly weaker than that of *E. coli*-type lipid A. Thus, the lipid A portion has been identified as the active center responsible for the majority of LPS-induced biological effects (19, 25). While the inner-core region has been reported to be essential to LPS's ability to induce leukotriene C_4 (20) and interleukin 1 (16) production by macrophages, there are also controversial reports that synthetic lipid A compounds are capable of inducing macrophages to produce interleukin 1 (9, 17, 18). There have been no other reports claiming participation of the polysaccharide portion in macrophage activation or other essential endotoxic activities.

The discovery of Toll-like receptors (TLRs) greatly advanced our understanding of the signal transduction mechanism of LPS. TLRs are mammalian homologues of the *Drosophila melanogaster* Toll protein. Among the TLRs identified so far, TLR4 has been reported to confer LPS responsiveness on LPS-unresponsive cells (1). TLR4 was initially identified as a molecule that increases constitutive but not LPS-inducible NF-κB activity. However, the finding of novel accessory molecule MD-2 (26), which confers LPS responsiveness on TLR4, and analyses of TLR4-deficient (8, 23, 24, 27, 31) mice have provided strong evidence for the involvement of TLR4 in LPS signaling. Based on these findings, the CD14/TLR4/MD-2 complex is now considered to be the predominant receptor for LPS.

We recently found that lipid A preparations from various *Salmonella* strains and synthetic *Salmonella*-type lipid A (516) exert very little stimulatory activity on human macrophages, although their LPS preparations and both lipid A and LPS preparations from *E. coli* are highly active (28). To clarify the basis of this phenomenon, in this study we investigated the role of the polysaccharide portion of LPS in TLR4-mediated activation of NF-κB, and the results showed that the polysaccharide portion of *Salmonella* LPS is indispensable to activation of NF-κB via human CD14/TLR4/MD-2. To our knowledge, this is the first report claiming participation of the polysaccharide portion in LPS-induced activation of NF-κB.

MATERIALS AND METHODS

Cell culture and reagents. Human embryonic cell line 293 (obtained from the Human Science Research Resources Bank, Tokyo, Japan) and mouse macrophage-like cell line RAW 264 (obtained from the Riken Cell Bank, Tsukuba, Japan) were grown in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Rockville, Md.) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (Gibco BRL), penicillin (100 U/ml), and streptomycin (100 μg/ml). Human monocyte-like cell line THP-1 (obtained from the Human Science Research Resources Bank) was grown in the same manner as the 293 cells except that RPMI 1640 (Gibco BRL) was used instead of DMEM. *Salmonella* LPS and its lipid A were prepared from *Salmonella enterica* serovar Abortusequi as described

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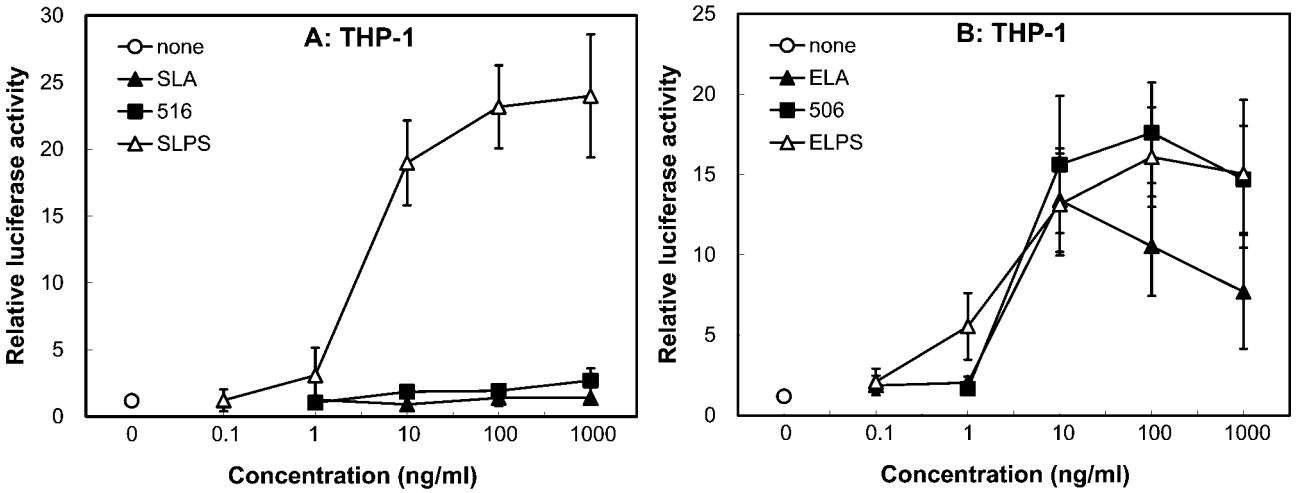

FIG. 1. Loss of the polysaccharide structure greatly reduces the activity of *Salmonella* LPS in human macrophage cells. Differentiated THP-1 cells were transiently transfected with an NF-κB-dependent luciferase reporter plasmid. After 24 h, cells were either unstimulated (open circles) or stimulated for 6 h with the LPS or lipid A preparations indicated in the presence of 10% (vol/vol) fetal calf serum, and luciferase activity was then measured. Values are means ± standard errors of the means from at least four independent experiments. ELA, *E. coli* lipid A; SLA, *Salmonella* lipid A; ELPS, *E. coli* LPS; SLPS, *Salmonella* LPS.

previously (28). *S. enterica* serovar Minnesota R595 LPS was prepared by the phenol-chloroform-petroleum ether method (4), and its lipid A was obtained by treating the LPS with 1% acetic acid for 90 min at 100°C (5). *E. coli* O111:B4 LPS and *E. coli* F-583 (Rd mutant) diphosphoryl lipid A were obtained from Sigma (St. Louis, Mo.). *E. coli* O111:B4 LPS was repurified according to the method described by Hirschfeld et al. (6). A polysaccharide preparation was obtained from *Salmonella* serovar Abortusequi LPS as described previously (28). Peptidoglycan from *Staphylococcus aureus* was obtained from Wako Pure Chemical Industries (Tokyo, Japan).

Expression plasmids. Plasmids containing human CD14 and mouse CD14 cDNAs were provided by Shunsuke Yamamoto (Medical College of Oita, Oita, Japan). The regions encoding human TLR4, mouse TLR4, and human MD-2 were amplified by reverse transcription-PCR (RT-PCR) from total RNA prepared from human spleen (OriGene Technologies, Rockville, Md.), murine fibroblast L929 cells, and THP-1 cells, respectively. The region encoding mouse MD-2 was amplified from a mouse embryo cDNA library (Clontech, Palo Alto, Calif.). Each PCR product was cloned into mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, Calif.). The coding regions of all constructs described above minus the coding sequences for their respective signal peptide sequences were subcloned into the downstream region of a modified pcDNA3 vector, in which the coding sequence for the preprotrypsin signal peptide sequence precedes that for the NH_2-terminal EIAV tag epitope (amino acid sequence: ADRRIPGTAEE). NF-κB-dependent luciferase reporter plasmid pELAM-L was described previously (22).

NF-κB reporter assay. The NF-κB-dependent luciferase reporter assay was performed as described elsewhere (22). Briefly, human embryonic kidney 293 cells (3×10^5 to 5×10^5/well) were plated in six-well dishes and on the following day were transfected by the calcium phosphate precipitation method with 0.2 μg of pELAM-L and 0.05 μg of pRL-TK (Promega, Madison, Wis.) for normalization. THP-1 cells (2×10^6/well) were plated in six-well dishes and differentiated with 100 ng of phorbol myristate acetate (Sigma)/ml plus 100 nM 1,25-dihydroxy vitamin D_3 (Wako Pure Chemical Industries). The cells were transfected 3 to 4 days later by using 3 μl of FuGene (Roche Diagnostics, Basel, Switzerland) with 1 μg of pELAM-L and 0.1 μg of pRL-TK (Promega) for normalization. RAW 264 cells (3×10^5 to 5×10^5/well) were plated in six-well dishes and on the following day were transfected by using 3 μl of FuGene with 0.5 μg of pELAM-L and 0.5 μg of pRL-TK for normalization. At 24 h after transfection, cells were stimulated for 6 h, and the reporter gene activity was measured according to the manufacturer's (Promega) instructions.

RESULTS

We recently discovered (28) that *Salmonella* lipid A preparations obtained from *Salmonella* serovar Abortusequi, *Salmonella* serovar Minnesota, and *S. enterica* serovar Typhimurium and synthetic *Salmonella*-type lipid A (compound 516) have very little ability to induce tumor necrosis factor alpha production or degradation of IκB-α in human macrophage cell lines (THP-1 and U937) but that the original LPS and lipid A preparations from *E. coli* showed strong ability to induce both. To confirm this phenomenon in an NF-κB-dependent reporter assay system, we first examined the activity of lipid A and LPS prepared from *Salmonella* serovar Abortusequi and *E. coli* on human macrophage cell line THP-1 (Fig. 1). In differentiated THP-1 cells neither *Salmonella* lipid A nor synthetic *Salmonella*-type lipid A (compound 516) significantly increased reporter activity at concentrations up to 1 μg/ml, whereas strong activation was observed in response to *Salmonella* LPS (Fig. 1A). By contrast, *E. coli* lipid A and synthetic *E. coli*-type lipid A (compound 506) strongly increased luciferase activity, and their activities were comparable to the activity of *E. coli* LPS (Fig. 1B). The failure of this *Salmonella* lipid A preparation to induce reporter activity was not due to improper preparation of lipid A because this lipid A preparation strongly activated reporter activity in mouse macrophage cell line RAW 264 (Fig. 2). Thus, our previous finding that *Salmonella* lipid A, but not *Salmonella* LPS, is inactive in human macrophages was confirmed in our NF-κB-dependent reporter assay system.

Since the CD14/TLR4/MD-2 complex is considered to be the predominant receptor for LPS, we next investigated the effect of lipid A and LPS preparations on NF-κB-dependent reporter activity in 293 cells transiently expressing CD14/TLR4/MD-2 (Fig. 3). In 293 cells transfected with a control vector, no activation of reporter activity in response to *Salmonella* LPS was observed (data not shown). In cells expressing CD14/TLR4/MD-2 of human origin, *Salmonella* LPS increased the reporter activity in a concentration-dependent manner, whereas the activity of *Salmonella* lipid A and 516 was much weaker than that of *Salmonella* LPS (Fig. 3A). As a control, the effects of *E. coli* LPS and lipid A in these cells were also

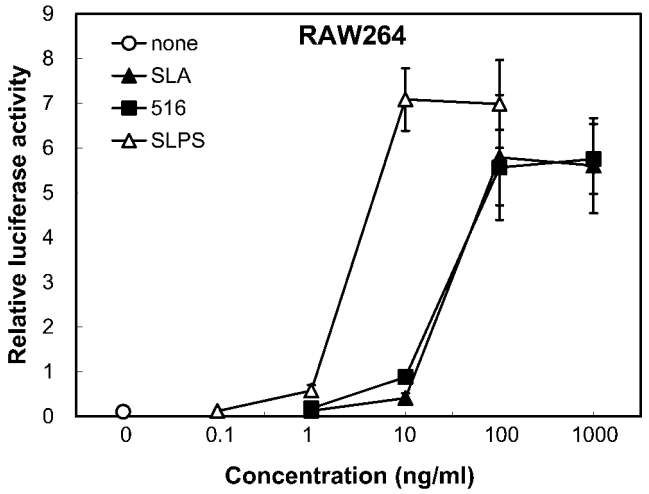

FIG. 2. The polysaccharide structure is not required for the activity of *Salmonella* LPS in RAW 264 cells. RAW 264 cells were transiently transfected with an NF-κB-dependent luciferase reporter plasmid. After 24 h, cells were either unstimulated (open circles) or stimulated for 6 h with the LPS or lipid A preparations indicated in the presence of 10% (vol/vol) fetal calf serum, and luciferase activity was then measured. Values are means ± standard errors of the means from four independent experiments. SLA, *Salmonella* lipid A; SLPS, *Salmonella* LPS.

examined. As observed in THP-1 cells, *E. coli* lipid A and 506 increased reporter activity and their activity was only slightly less than that of *E. coli* LPS (Fig. 3B). These results indicate that the polysaccharide structure plays an indispensable role in *Salmonella* LPS-induced activation of NF-κB through human CD14/TLR4/MD-2.

To investigate which part of the polysaccharide of *Salmonella* LPS is involved in its activity, we next examined the effect of LPS prepared from a *Salmonella* serovar Minnesota Re mutant (Fig. 4) whose polysaccharide structure consists of only a disaccharide composed of 2-keto-3-deoxyoctonic acid. The LPS preparation increased reporter activity with a potency similar to that of *E. coli* LPS (Fig. 1 and 3) in all differentiated THP-1 cells (Fig. 4A) and 293 cells expressing CD14/TLR4/MD-2 (Fig. 4B and C). Lipid A prepared from Re LPS only weakly activated THP-1 cells and 293 cells expressing human CD14/TLR4/MD-2 (Fig. 4A and B), whereas, in 293 cells expressing mouse CD14/TLR4/MD-2, the lipid A preparation showed a strong activity, which was comparable to that of Re LPS (Fig. 4C). This finding indicates that the presence of only two molecules of 2-keto-3-deoxyoctonic acid is sufficient to increase the activity of *Salmonella* lipid A. A polysaccharide preparation alone obtained from our *Salmonella* serovar Abortusequi LPS did not increase reporter activity even at a concentration of 10 μg/ml, and no increase in reporter activity was observed in response to 516 when both the polysaccharide preparation (10 μg/ml) and 516 (1 μg/ml) were added to 293 cells expressing human CD14/TLR4/MD-2 (data not shown). This indicates that the polysaccharide portion itself does not possess intrinsic activity.

Since the activity of our *Salmonella* LPS preparations may have been attributable to a contaminant(s) that activates NF-κB through TLR2, as reported by Hirschfeld et al. (6) and Tapping et al. (29), we examined the effect of our *Salmonella* LPS preparations on 293 cells transiently expressing CD14 and TLR2. Peptidoglycan from *Staphylococcus aureus* strongly increased the reporter activity in these cells, whereas no significant activation was observed in response to LPS from *Salmonella* serovar Abortusequi or Re LPS from *Salmonella* serovar Minnesota at concentrations up to 10 μg/ml (data not shown), indicating that no TLR2-stimulating contaminants were present in our *Salmonella* LPS preparations. Taken together, these results indicate that the polysaccharide structure plays an indispensable role in *Salmonella* LPS-induced activation of NF-κB through human CD14/TLR4/MD-2.

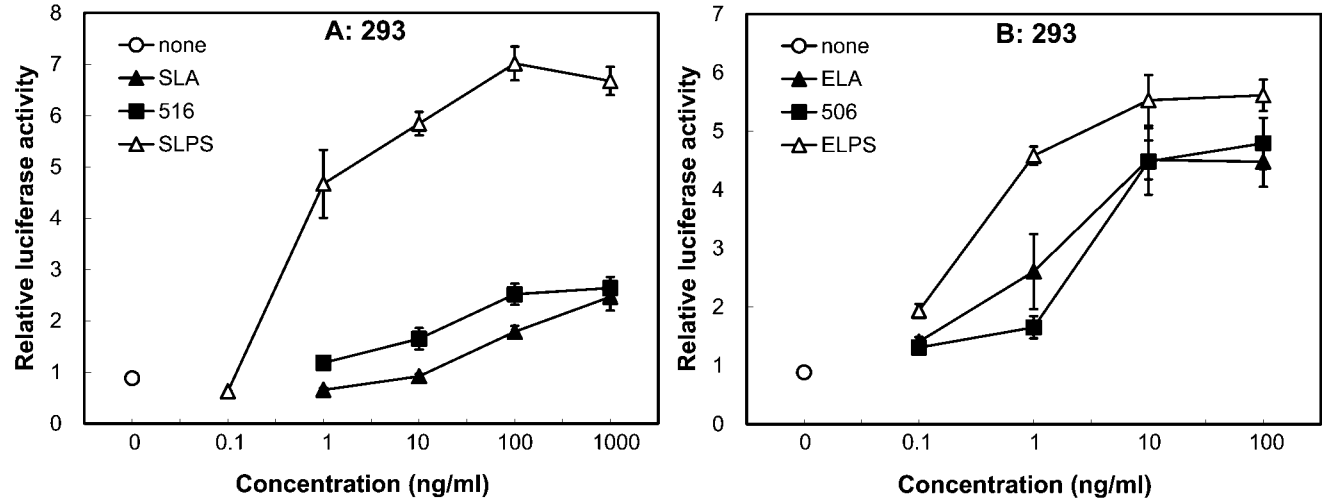

FIG. 3. Loss of the polysaccharide structure greatly reduces the activity of *Salmonella* LPS in 293 cells expressing human CD14/TLR4/MD-2. 293 cells were transiently transfected with CD14 (0.1 μg), TLR4 (2 ng), and MD-2 (2 ng) plasmids of human origin, together with an NF-κB-dependent luciferase reporter plasmid. After 24 h, cells were either unstimulated (open circles) or stimulated for 6 h with the LPS or lipid A preparations indicated in the presence of 10% (vol/vol) fetal calf serum, and luciferase activity was then measured. Values are means ± standard errors of the means from at least four independent experiments. ELA, *E. coli* lipid A; SLA, *Salmonella* lipid A; ELPS, *E. coli* LPS; SLPS, *Salmonella* LPS.

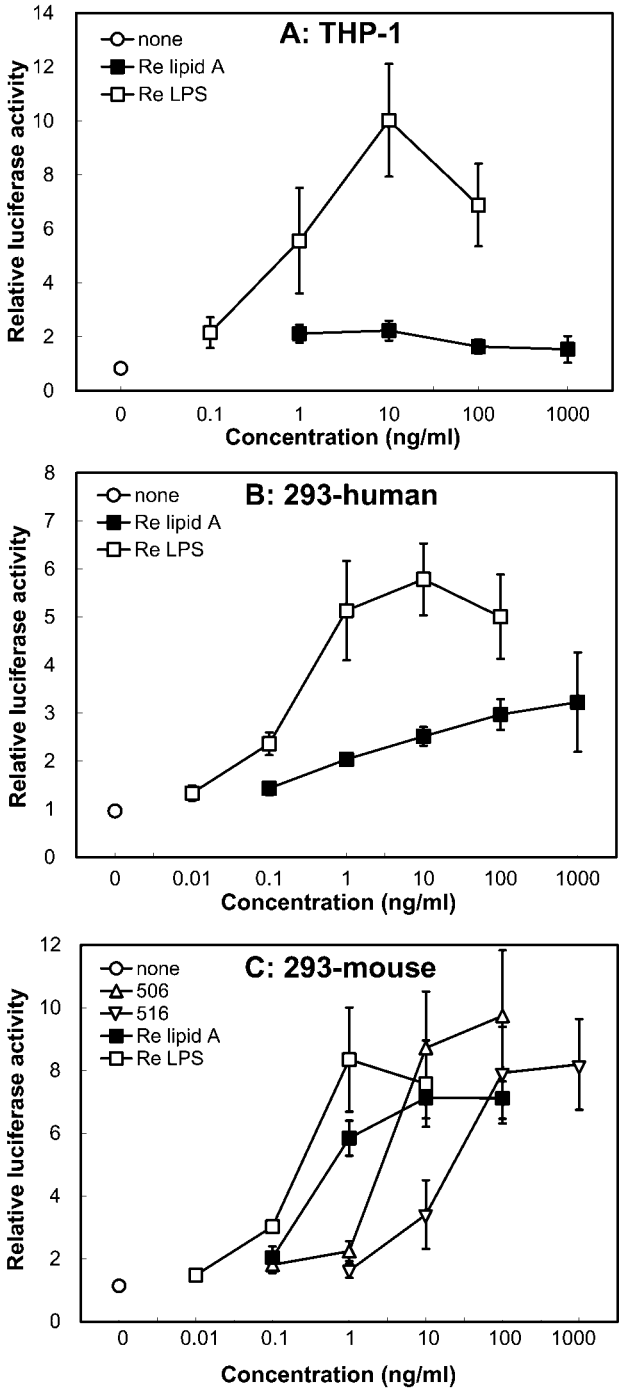

FIG. 4. Presence of only a disaccharide is sufficient to increase the activity of *Salmonella* lipid A in differentiated THP-1 cells and 293 cells expressing human CD14/TLR4/MD-2. Differentiated THP-1 cells (A) were transiently transfected with an NF-κB-dependent luciferase reporter plasmid. 293 cells were transiently transfected with CD14 (0.1 μg), TLR4 (2 ng), and MD-2 (2 ng) plasmids of either human (B) or mouse (C) origin, together with an NF-κB-dependent luciferase reporter plasmid. After 24 h, cells were either unstimulated (open circles) or stimulated for 6 h with the LPS or lipid A preparations indicated in the presence of 10% (vol/vol) fetal calf serum, and luciferase activity was then measured. Values are means ± standard errors of the means from at least three independent experiments.

DISCUSSION

Although it is widely accepted that the lipid A moiety is responsible for the biological activity of LPS, we recently found that lipid A preparations from various *Salmonella* strains exert very little stimulatory activity on human macrophages, even though their LPS preparations are highly active (28). There have been no studies reporting that the polysaccharide structure covalently attached to lipid A is required for LPS-induced activation of NF-κB. The possibility of improper preparation of lipid A can be ruled out because the lipid A preparations were highly active in mouse macrophages and because synthetic *Salmonella*-type lipid A (compound 516), which is highly active in mouse macrophages, also showed very little activity in human macrophages (28; this study). The finding that *Salmonella* lipid A and 516 possess strong activity in mouse macrophages also indicates that the lack of activity of these lipid A preparations in human macrophages is not simply due to the hydrophobic nature of the lipid A molecules. The possibility that only a specific cell line of human origin, such as the THP-1 line used in this study, shows limited sensitivity to *Salmonella* lipid A is also unlikely because another human macrophage cell line, U937, also failed to respond to *Salmonella* lipid A (28) and because 293 cells expressing CD14/TLR4/MD-2 of human origin showed limited sensitivity to *Salmonella* lipid A (Fig. 3A). All of these findings support the concept that the polysaccharide portion is indispensable to *Salmonella* LPS-induced activation of NF-κB through human CD14/TLR4/MD-2. Although a number of studies have shown that lipid A preparations of *Salmonella* origin activate macrophages of human origin, we found (28) that some *Salmonella* lipid A preparations still contain considerable amounts of *E. coli*-type lipid A as a major component that is biosynthesized depending on the bacterial culture conditions and that is active in human macrophages. Therefore, the activity of *Salmonella* lipid A preparations of bacterial origin needs to be evaluated with caution.

The only structural difference between the *E. coli*-type (compound 506) and *Salmonella*-type (compound 516) lipid A molecules is a hexadecanoyl acid moiety attached to the hydroxyl residue of 3-hydroxytetradecanoic acid bound to position 2 of reducing diglucosamine (28). The activities of these lipid A compounds in human macrophages, but not mouse macrophages, were dramatically different, suggesting that human macrophages are capable of discriminating their structural differences. We recently found that species differences in MD-2 molecules are involved in this species-specific action of *Salmonella* lipid A (21). By contrast, there was no significant difference between the activities of the *E. coli* and *Salmonella* LPS preparations. The chemical structure of the polysaccharide portion of LPS varies greatly among different bacterial species. However, it is unlikely that a polysaccharide specific to *Salmonella* LPS possesses activity that induces NF-κB, because *Salmonella* serovar Minnesota Re LPS, whose structure consists of lipid A and two molecules of 2-keto-3-deoxyoctonic acid, a saccharide commonly found both in *E. coli* and *Salmonella* LPS, also strongly activated NF-κB (Fig. 4). Moreover, a polysaccharide preparation obtained from our *Salmonella* LPS by itself induced neither tumor necrosis factor alpha production nor NF-κB activation in THP-1 cells and 293 cells expressing

human CD14/TLR4/MD-2, even when the polysaccharide preparation and *Salmonella* lipid A were added together (28; this study). Therefore, *Salmonella* LPS requires at least a disaccharide structure covalently bound to lipid A for its activity.

Use of *Salmonella* lipid A preparations whose major lipid A structure consists of typical *Salmonella*-type hepta-acylated lipid A molecules in addition to synthetic *Salmonella*-type lipid A allowed us to identify the essential role of the polysaccharide portion of *Salmonella* LPS in macrophage activation. The concept that the active center of LPS resides in its lipid A portion is well established; however, the activity of LPS and that of its lipid A have not been systematically compared until now. Although we cannot generalize the need for the polysaccharide portion to other types of LPS, the essential feature of the polysaccharide portion may not be restricted to *Salmonella* LPS. The function of the polysaccharide portion in the interaction of LPS molecules with the LPS receptor molecules is unknown. Since the free polysaccharide itself did not activate macrophages even when stimulated in combination with *Salmonella* lipid A, covalently bound polysaccharide seems to change the conformation of LPS, resulting in a change in its binding mode or binding site at LPS receptor molecules. Because of this, *Salmonella* LPS and lipid A may serve as useful tools to identify the part of LPS and the molecule of the LPS receptor complex that are involved in LPS signal transduction in macrophages.

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